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Transcriptome analysis suggests a role for the differential expression of cerebral aquaporins and the MAPK signalling pathway in human temporal lobe epilepsy

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Abstract

Epilepsies are common disorders of the central nervous system (CNS), affecting up to 2% of the global population. Pharmaco-resistance is a major clinical challenge affecting about 30% of temporal lobe epilepsy (TLE) patients. Water homeostasis has been shown crucial for regulation of neuronal excitability. The control of water movement is achieved through a family of small integral membrane channel proteins called aquaporins (AQPs). Despite the fact that changes in water homeostasis occur in sclerotic hippocampi of people with TLE, the expression of AQPs in the epileptic brain is not fully characterised. This study uses microarray and ELISA methods to analyse the mRNA and protein expression of the human cerebral AQPs in sclerotic hippocampi (TLE-HS) and adjacent neocortex tissue (TLE-NC) of TLE patients.

The expression of AQP1 and AQP4 transcripts was significantly increased, while that of the AQP9 transcript was significantly reduced in TLE-HS compared to TLE-NC. AQP4 protein expression was also increased while expression of AQP1 protein remained unchanged, and AQP9 was undetected.

Microarray data analysis identified 3,333 differentially regulated genes and suggested the involvement of the MAPK signalling pathway in TLE pathogenesis. Proteome array data validated the translational profile for 26 genes and within the MAPK pathway (e.g. p38, JNK) that were identified as differentially expressed from microarray analysis. ELISA data showed that p38 and JNK inhibitors decrease AQP4 protein levels in cultured human primary cortical astrocytes. Elucidating the mechanism of selective regulation of different AQPs and associated regulatory proteins may provide a new therapeutic approach to epilepsy treatment.
Introduction

Epilepsies are among the most common disorders of the central nervous system (CNS). It is estimated that over 65 million people worldwide suffer from these debilitating conditions (Ngugi et al., 2010; Moshé et al., 2015). Temporal lobe epilepsy (TLE) accounts for about one-third of all patients with epilepsy (Heuser et al., 2012). The majority of current anti-epileptic drugs (AEDs) target the ion channels that mediate neuronal excitability (Rogawski & Löscher, 2004; Landmark, 2006), however about 30% of patients become medically refractory to AEDs (Löscher, 2011; Löscher et al., 2013). Pharmaco-resistance in TLE is often associated with hippocampal sclerosis (HS). The sclerotic hippocampus is characterized by neuronal loss, astrogliosis and increased microvascular density (Blümcke et al., 2012; Alonso-Nanclares & DeFelipe, 2014), particularly at the perivascular end-feet of astrocytes (Eid et al., 2005).

Astrocytes play a major role in regulating water and ion (particularly K\(^+\)) homeostasis in the brain. The association of K\(^+\) homeostasis and water transport is known to mediate synaptic transmission by modulating the extracellular environment around neurons (Simard & Nedergaard, 2004); additionally astrocytes can be depolarized and produce an action potential exhibiting neuronal-like characteristics (Bordey & Sontheimer, 1998). Water homeostasis is an important factor in modulating seizure susceptibility (Andrew, 1991; Schwartzkroin et al., 1998; Lee et al., 2012). Magnetic resonance imaging (MRI) of sclerotic hippocampi from TLE sufferers (TLE-HS) showed an increase in T2-weighted signal (Bronen et al., 1991; Dawe et al., 2014) and diffusion-weighted imaging demonstrated higher diffusion coefficient in TLE-HS patients compared to controls (Wiesmann et al., 1999). All these data indicated higher free water content in sclerotic hippocampi tissue (Lee et al., 2004; Heuser et al., 2010).

The control of water movement into and out of cells is achieved via a family of small integral membrane proteins called aquaporins (AQP). Thirteen human AQP (AQP 0-12), have
been identified and characterised (Day et al., 2014). AQP4 is the predominant AQP in the CNS and it has been identified and characterised in both neurons (Binder et al., 2006) and glia (Nielsen et al., 1997). In the human hippocampus, AQP4 has been found in the Cornu Ammonis (CA) and dentate gyrus (DG) areas. At the cellular and subcellular level, AQP4 is abundantly expressed in the plasma membrane of the astrocytes that sheath the glutamatergic synapses, and shows the highest expression in perivascular astrocytes, where it is localised to the plasma membrane of astrocytic end-feet at the glia limitans (Lee et al., 2004; Gleiser et al., 2016). AQP4 is co-localized with inwardly-rectifying K+ channels (Kir4.1) and glial K+ uptake is attenuated in AQP4 knockout mice compared to wild-type, indicating a functional interaction (Padmawar et al., 2005; Binder et al., 2006; Binder et al., 2012). In the sclerotic hippocampus, astrocytes have fewer Kir4.1 channels and immunohistological studies have indicated that Kir4.1 is lost from perivascular end-feet in sclerotic hippocampi of TLE patients (Heuser et al., 2012). It is not known if this change in expression is due to; or driven by, changes at the transcript level or changes in protein stability/degradation. There is also some controversy in the literature concerning the exact nature of the relationship between AQP4 and Kir4.1 in astrocytes, with one study suggesting that glial AQP4 and Kir4.1 do not interact functionally (Zhang & Verkman, 2008).

Other AQPs have been identified in the CNS: AQP1 in the dorsal-root ganglia (Shields et al., 2007) and in the epithelium of the choroid plexus, where cerebrospinal fluid (CSF) is produced (Oshio et al., 2005) and AQP9 in the substantia nigra (Badaut et al., 2004). AQP1, 3, 4, 5, 8, 9 and 11 have been shown to be expressed at gene and protein levels in the rodent brain, principally in astrocytes (Gorelick et al., 2006; Yang et al., 2009; Badaut et al., 2014). Moreover, AQPs 3, 5 and 8 are expressed in the DG area in both astrocytes and neurons (Yang et al., 2009). However, there is still little known about the expression profile of human cerebral AQPs, particularly in TLE patients. For example, there is contradictory evidence describing the expression of AQPs in sclerotic and non-sclerotic hippocampi: Lee et al showed AQP1 and AQP4 protein expression in astrocytes located in the hippocampal tissues, but only AQP4 protein expression was found to be upregulated in perivascular
astrocytes in the sclerotic hippocampi tissue of TLE patients (n=4) compared to non-sclerotic hippocampi (n=5). Both Lee et al, and Eid et al. reported an increase in AQP4 protein in mesial temporal lobe epilepsy (MTLE) hippocampi compared to non-MTLE, however a simultaneous loss of perivascular AQP4 localization was seen and attributed to loss of dystrophin localization. Bebek et al. reported that there was neither difference in the expression of AQP1 and AQP4 transcripts nor the AQP4 protein in 23 patients. Jamali et al. showed an increase in AQP1 transcript following a microarray study on hippocampal tissues from 15 TLE patients; however, their findings were not consistent following RT-PCR validation and therefore the gene was discounted from further analysis.

A number of studies have investigated the possible mechanisms involved in AQP regulation; these studies identified the p38 Mitogen-activated Protein Kinase-dependent (MAPK) pathway as the possible primary mechanism controlling the altered expression of some AQPs (Fujita et al., 2003; Yang et al., 2013b). The MAPK family consists of three major pathways, the extracellular signal regulated kinase (ERK) pathway, the p38 pathway and C-Jun N-terminal kinases (JNK) pathway. The MAPK pathway has been implicated in increased epileptic seizures due to hippocampal sclerosis (Pernice et al., 2016). The purpose of this current study was to analyse gene expression and protein profiling in TLE-HS and the non-sclerotic temporal lobe that had no ictal or inter-ictal activity (TLE-NC; neocortex). Gene network analysis using Database for Annotation, Visualization, and Integrated Discovery (DAVID) and the Kyoto Encyclopedia of Genes and Genomes (KEGG) analysis identified the MAPK pathway as the most likely pathway affected in TLE identified in the microarray. ELISA was conducted to investigate the involvement of p38, ERK1/2 and JNK MAPKs in regulating AQP4 protein expression in primary human astrocytes using the specific inhibitors: SB203580, PD98059 and SP600125, respectively (Wang et al., 2007). Direct targeting of AQPs and/or the molecular mechanisms of their regulation via the MAPK signalling pathways could open new horizons for more specific and targeted treatments for TLE.
Materials and methods:

Sample collection and patients’ clinical information:

This research was approved by South Yorkshire research ethics committee (08/H1310/49) and it followed the code of ethics of the World Medical Association (2001). The samples were obtained from the Royal Hallamshire Hospital (R&D approval STH15210). All samples were obtained with the understanding and the written consent of each patient. The sample collection procedure fully conformed to the Code of Ethics of the World Medical Association (Declaration of Helsinki), British Medical Journal (1964).

Patients with pharmaco-resistant TLE associated with unilateral hippocampal sclerosis were recruited. The diagnosis of TLE-HS was made by the treating clinician based on MRI brain scan and inter-ictal and ictal EEG characteristics, being consistent with a seizure focus in the hippocampus within the temporal lobe. The total numbers of patients used for this study were ten TLE patients (6 females and 4 males), their age at surgery was 38.2 ± 10.2 years, the average age at onset of first non-febrile seizure was 11.6 ± 9.9 years and the average duration of epilepsy was 26.6 ± 15.8 years. These patients underwent a therapeutic selective amygdalohippocampectomy. After surgery, two samples were obtained: sclerotic hippocampus (TLE-HS), and non-spiking neocortex (TLE-NC). Full clinical information for the patients is shown in Table 1.

Microarray analysis (MA)

Total RNA was extracted using SV Total RNA Isolation System (Promega, Z3100) according to the manufacturer’s instructions. The quality and purity of the prepared RNA was assayed using a NanoDrop-1000 spectrophotometer and Agilent Bioanalyser-2100, to ensure only high-quality RNA samples with A_{260}/A_{280} ratio of 1.8 to 2.0 and an A_{260}/A_{230} ratio of > 2.0 were used for the microarray study. Starting from 100 ng of RNA, cRNA samples were synthesized and labelled with Cy3 or Cy5 using the Two-colour Low Input Quick Amp labelling kit (Agilent Technologies, 5190-2306). TLE-HS hippocampi were labelled with Cy5 and TLE-NC samples were labelled with Cy3. Equal amounts of the labelled TLE-HS and
TLE-NC samples were co-hybridized onto SurePrint G3 Human Gene Expression 8x60K Microarrays (Agilent Technologies, G4851A). The microarray array was scanned on Agilent Technologies SureScan scanner. Raw data was extracted using Agilent feature extraction software (version 10.7.1) and was then normalized by the locally weighted scatter plot smoothing (LOWESS) normalization method. The Rank Product (RP) test was used to identify the differentially expressed genes ($P < 0.05$) using Multi-Experiment Viewer (MeV) software, version 4.9 (Saeed et al., 2003; Breitling et al., 2004; Koziol, 2010). The list of up- and down-regulated genes was submitted to the bioinformatics and functional annotation tool provided by DAVID, version 6.7 (Huang et al., 2009a; b). Then KEGG pathway enrichment analysis was done to identify potential pathways that are possibly associated with TLE-HS pathophysiology (http://david.abcc.ncifcrf.gov/).

In order to obtain biological information underpinning the molecular mechanisms and regulatory networks associated with TLE pathogenesis, microarray data was further assessed using the “TargetMine” (http://targetmine.mizuguchilab.org/) and the Gene Ontology Consortium (http://geneontology.org/) bioinformatics tools to obtain functional annotation clustering and gene ontology (GO) terms for the differentially-regulated genes (DEGs). Key results are summarised along with gene count, p value and fold enrichment in supplementary Table 1.

**Sandwich enzyme-linked immunosorbent assay (ELISA)**

A 96-well microtiter flat-bottomed polystyrene plate (Nunc, Wiesbaden, Germany) was coated by overnight incubation at 4°C with 5 µl/well of either rabbit polyclonal anti-AQP4 (Abcam, ab46182) diluted 1:500, rabbit polyclonal anti-AQP1 (Santa Cruz, sc-208110) diluted 1:400 or rabbit polyclonal anti-AQP9 (LS-C20770) diluted 1:500; all antibodies were diluted in carbonate/bicarbonate buffer (pH 9.6). The plates were washed twice for 5 minutes with Phosphate Buffered Saline and 0.05% Tween 20 (PBS-T) (pH 7.5). The remaining unsaturated protein-binding sites in the coated wells were blocked by adding blocking buffer (5% non-fat dry milk/PBS) and then incubated overnight at 4°C with gentle shaking. Plates
were washed twice for 5 minutes with PBS-T. Hippocampal proteins were extracted using Cellytic™ (Sigma, Cat. No. C2978) supplemented with protease inhibitor cocktail (Sigma, Cat. No. P2714, 1:100). The total protein concentration was determined using Pierce™ BCA Protein Assay Kit (Thermofisher Scientific, Cat. No. 23225) following the manufacturer’s procedure. 100 μl equally diluted samples at a concentration of 600 mg/ml were added to each well and incubated for 90-120 minutes at 37°C. Samples were then aspirated and the plates were washed twice with PBS-T. 100 μl of either the 1:1 diluted mouse monoclonal anti-AQP4 antibody (Abcam, ab9512), mouse monoclonal anti-AQP1 (Abcam, ab11025), or goat polyclonal anti-AQP9 (Santa Cruz F-17; sc-14988) were added to each well. The plates were covered with adhesive plastic and incubated for 2 h at 37°C and then washed twice for 5 minutes with PBS-T. Then 100 μl of horseradish peroxidase (HRP)-conjugated secondary antibody, either chicken anti-mouse (Santa Cruz, sc-2954) for AQP4 and AQP1 or chicken anti-goat (c-2953) for AQP9, diluted at 1: 5,000 in freshly-prepared blocking buffer, was added to each well and incubated for 30 minutes at 37°C. The plates were washed (with gentle shaking) four times for 5 minutes with 200 μl PBS-T, followed by a single wash with PBS. The plates were incubated with 100 μl/well of RayBio™ TMB One-Step Substrate Reagent (Raybiotech; Cat. No. J120215098), at room temperature for 30 minutes, under light-protected conditions After the colour was developed, the reaction was stopped by adding 50 μl of 2 M H₂SO₄. The absorbance values were then immediately measured at 450 nm using a Perkin Elmer Wallac 1420 Victor2 microplate reader.

**Proteome Profiling**

Proteome Profiler™ Human MAPK array (R&D Systems, ARY002B) was used to investigate the possible role of signaling molecules of all three major families of MAPKs, the extracellular signal-regulated kinases (ERK1/2), c-Jun N-terminal kinases (JNK1-3), and different p38 isoforms (α/β/δ/γ) in TLE-HS. Protein from 3 matching pairs of TLE-HS and TLE-NC patient samples were extracted using CelllyticTM 10ml per gram of tissue (Sigma, C2978) supplemented with protease inhibitor cocktail 1:100 (Sigma, P2714). The total protein was quantified using Pierce™ BCA protein assay kit (Thermofisher scientific, 23225)
following the manufacturer's procedure. Each proteome profiler membrane was then incubated with 300 μg of protein lysate, according to the manufacturer's instructions. The HRP-conjugated streptavidin provided in the kit was replaced with IRDye® 800CW Streptavidin (LI-COR, 926-32230) and it was diluted at 1:2000 using the array buffer 5 (R&D Systems, ARY002B). All of the following steps were performed according to the manufacturer's recommendation. The arrays were scanned with LI-COR Odyssey® Infrared Imaging System and quantified with Image Studio™ software (LI-COR) to determine the relative amount of the specific MAPK proteins.

Cell Culture
Primary human cortical astrocytes (Sciencell, Cat. No. 1800) were plated into 75 cm² culture flasks (Thermo Scientific Nunc Cell Culture Treated EasyFlasks) and cultured routinely in Astrocyte Medium (Sciencell; 1801) containing 1% fetal bovine serum (FBS, Sciencell Cat. No. 0010), 5 ml astrocyte growth supplement (AGS, Sciencell Cat. No. 1852), and 5 ml 1% penicillin/streptomycin solution (P/S, Sciencell Cat. No. 0503). Cultures were maintained at 37°C and 5% air in a humidified environment.

Treatment with p38, ERK1/2 and JNK inhibitors
In order to investigate the possible role of p38, ERK1/2 and JNK in the differential regulation of AQP4 expression in astrocytes, the effects of SB20358010 μM a p38 inhibitor (Hua et al., 2002), PD98059 10 μM a ERK1/2 inhibitor (Hua et al., 2002) and SP600125 40 μM a JNK inhibitor (Wallace et al., 2012) were examined in human primary cortical astrocytes for 6h. Data are presented as a fold-change normalised to the experimental control (n=4). In all cases, cells were at least 92% viable.

Statistical analysis
Microarray analysis, ELISA and proteome profiling data were found not to be normally distributed using a Shapiro-Wilk test, therefore Kruskal-Wallis with Conover–Inman post hoc
analysis tests were used to identify significant differences between samples ($P \leq 0.05$ was taken as significant).

**Results**

**Microarray (MA) gene expression analysis**

In the MA analysis on the SurePrint G3 human gene expression 60K microarrays (Agilent Technologies, G4851A), a total number of 34,197 uniquely-annotated probes were present and significant differences in the expression levels of 3,333 genes were identified. In the TLE-HS tissue, 1,821 genes (5% of all detected genes) were significantly up regulated and 1,511 genes (4%) were significantly down regulated compared to TLE-NC tissue from seven patients. Figure 1 shows the data for all investigated cerebral AQPs and their associated Kir4.1 channel. Fold change (FC) was calculated by dividing the value representing the gene expression in TLE-HS by the corresponding TLE-NC value. AQP1 and AQP4 mRNA levels were both significantly increased (4.03-fold ± 0.89, $p<0.0001$ (n= 7) and 3.42-fold ±0.58, $p<0.0001$ (n= 7); respectively). AQP9 mRNA levels were significantly reduced to 0.31-fold ±0.06, $p=0.01$ (n= 7). There was no significant difference in mRNA levels for AQP3, AQP5, AQP8, AQP11 or Kir4.1.

**Protein expression levels of AQP4 and AQP1**

The AQP ELISA data demonstrated a significant increase in AQP4 protein expression in TLE-HS samples (2.53-fold ± 0.1-fold, $p<0.0001$ n= 3) compared to TLE-NC samples from the same set of patients (Figure 2A). AQP1 protein was measurable in both samples, however there was no detectable significant difference ($p=0.065$) in AQP1 protein levels in the sclerotic TLE-HS samples compared to non-sclerotic TLE-NC samples (Figure 2B). The expression of AQP9 protein could not be detected using our custom-made ELISA system with the combination of antibodies used.

**DAVID/KEGG pathway analysis of MA data**
The 3,333 differently-regulated genes from the MA analysis were submitted to the bioinformatics database, DAVID, using KEGG database for pathway enrichment analysis. To minimize false positives among significantly-enriched functions, a false discovery rate (FDR) ≤0.05 (-log P-value = 1.33) was used to determine the probability that each biological function assigned to that data set was due to chance alone. The calcium signalling pathway, MAPK signalling pathway and neuroactive ligand-receptor interaction pathway showed the highest enrichment scores in the sclerotic TLE-HS tissue compared to TLE-NC (Figure 3). The MAPK signalling pathway was the second most highly-represented pathway with an enrichment score of 6.1 (after the Ca\(^{2+}\) signalling pathway with an enrichment score of 8.7) and the one with the highest number of significantly-regulated genes. The expression of 50 MAPK genes was altered: 27 genes had a higher expression and 23 genes had a lower expression in TLE-HS compared to the TLE-NC tissue. Their distribution profile within the MAPK pathway is illustrated in Figure 4.

**MAPK signalling profile in TLE-NC and TLE-HS samples**

Since analysis of the MA data demonstrated the involvement of the MAPK signalling pathway in TLE pathophysiology (Figures 3 and 4), 26 of the significantly-enriched MAPK related genes (Figure 5a) were investigated at the protein level using a Proteome Profiler Human MAPK Array Kit (Figure 5b). Transcription levels were determined by reanalysing the MA data for each of these 26 individual genes to determine the protein expression level in TLE compared to TLE-NC. In addition, the transcriptional and translational expression FC of a number of significant MAPKs was calculated by dividing the gene or protein expression level in TLE-HS by its corresponding TLE-NC value as shown in Table 2.

The results show significant up-regulation of ERK2 and MMK3 at both the gene (2.75-fold ±0.02; p<0.0001; 1.15-fold ±0.09; p<0.0001) and protein level (2.33-fold ±0.65; p=0.02; 2.26-fold ±0.53; p=0.02). There were also significant increases in protein levels of AKT Pan (3.78-fold ±0.48; p=0.0213); p53 (2.89-fold ±0.47; p=0.021) and RSK2 (1.75-fold ±0.28; p=0.02)
although there were no significant differences at the mRNA level. Several genes showed significant expression changes that were not accompanied by a corresponding change in protein expression. These included the up-regulation of ERK1 (1.34-fold ± 0.02; p<0.0001), GSK3-beta (1.81-fold ± 0.01; p<0.0001), HSP27 (2.01-fold ± 0.27; p=0.0062) and RSK1 (1.03-fold ± 0.01; p<0.018) and the down-regulation of GSK3-alpha (0.67-fold ± 0.06; p=0.0419), JNK2 (0.54-fold ± 0.04; p=0.0062) and p38 delta (0.66-fold ± 0.01; p=0.0033).

Effect of p38, ERK1/2 and JNK inhibitors on AQP4 protein expression in primary human cortical astrocytes using sandwich ELISA.

Data are presented as fold-change compared to untreated astrocytes (n=4). Figure 6 shows that treating astrocytes with the p38 inhibitor caused a significant reduction in AQP4 protein expression (0.67-fold ± 0.01; p=0.014). A similar trend in reduction of AQP4 protein expression was seen after treating the cells with a JNK inhibitor (0.57-fold ± 0.009; p=0.007). There was no change in AQP4 protein expression in astrocytes following ERK1/2 inhibitor treatment (p value=0.27).

Discussion

This study has demonstrated a differential expression of AQP1, 4 and 9 transcripts, and revealed changes in AQP4 protein expression in human TLE-HS along with twelve key regulatory elements of the MAPK pathway by DAVID/KEGG analysis. Our results together with evidence of TLE-impaired water homeostasis (Bronen et al., 1991; Lee et al., 2004; Heuser et al., 2010; Dawe et al., 2014) may implicate these elements in the pathophysiology of TLE directly or indirectly through their effect on AQP expression.

Regulation of water transport is increasingly being suggested as a mechanism in the aetiology of TLE (Bronen et al., 1991; Lee et al., 2004; Heuser et al., 2010; Dawe et al., 2014). A rapid increase in brain water-content can result in seizures (Andrew, 1991). Moreover, it has been suggested that seizures could cause cell swelling and a decrease in
the size of the extracellular space (Janigro & Walker, 2014). This can result in an increase in
the extracellular K\(^+\) concentration, which has a significant effect in increasing seizure
susceptibility (Dietzel et al., 1980; Dudek & Rogawski, 2005). Experiments in AQP4 \(-/-\) mice
suggested a direct role for AQP4 in controlling neurotransmission via modulating dopamine
metabolism (Fan et al., 2005; Ding et al., 2007) and glutamate uptake (Li et al., 2012; Yan et
al., 2013). Since elevated extracellular potassium and glutamate concentrations are well-
known drivers of epilepsy (Cho, 2013) and AQP4 appears to positively regulate the uptake of
both of these molecules into astrocytes, it may be that the upregulation of AQP4 is a
homeostatic response to high concentrations of these molecules. However, because AQP4
is mislocalized in the astrocytes of the sclerotic hippocampus (Eid et al., 2005; Alvestad et
al., 2013), this homeostatic response is insufficient for preventing seizures.
In the present study, the whole-genome transcriptional profile was determined using
microarray and the gene network analysing software DAVID and KEGG. Differentially
expressed putative target genes, biomarkers and pathways that may play a role in TLE have
been identified. This study reports that the transcript expression of AQP\(_1\) and 4 are
significantly increased, while the transcript expression of AQP9 is significantly reduced in
TLE-HS compared to TLE-NC. This was accompanied by a significant upregulation of the
transcripts of the astrocytic biomarker, glial fibrillary acidic protein (GFAP) in TLE-HS
compared to TLE-NC, which reflects a state of gliosis in TLE-HS (Lee et al., 2004). The
transcripts of the neuronal biomarker, neuronal nuclear antigen (NeuN), was significantly
decreased in TLE-HS compared to TLE-NC which could refer to neuronal loss in sclerotic
tissue samples (supplementary material Figure 1).

Of the investigated cerebral AQP genes, transcriptome analysis indicated only a significant
differential expression of AQP1, AQP4 and AQP9 transcripts. The ELISA data in this study
showed a significant increase in AQP4 protein expression in sclerotic tissue samples
compared to the non-sclerotic samples from the same set of patients that followed the same
trend of upregulation as seen at the mRNA level. This result is in qualitative agreement with
the findings of Das et al. (2012) who reported a ~1.8-fold increase in AQP4 protein expression in six sclerotic samples compared to three non-matched post-mortem controls. Similarly, Lee et al. (2004) reported a 1.6-fold increase in AQP4 transcript by microarray and a 2.6-fold increase by qPCR in sclerotic hippocampi from four mesial TLE patients (MTLE) compared to three non-matched TLE patients. This is qualitatively consistent with our observation of a 3.4-fold increase in AQP4 transcript by microarray in seven sclerotic hippocampi from TLE patients compared to matched non-sclerotic tissue. A study by Eid et al. reported a 3.6-fold increase in AQP4 protein in six MTLE patient hippocampi compared to six non-MTLE controls, and a simultaneous loss of perivascular AQP4 localization attributed to loss of dystrophin localization. Bebek et al. claimed that there was no change in AQP4 protein expression in five MTLE patients compared to five non-MTLE using immunofluorescence. However, the number of AQP4-positive cells per field was the reported measure for AQP4 expression, but there was no attempt to measure changes in intensity of AQP4 staining in those positive cells. In addition, the authors reported that AQP4 transcript was unchanged in 23 MTLE samples compared to seven post-mortem controls. However, their control samples spanned a 1 million-fold range (normalised expression from $10^{-7}$ to $10^{-1}$; (see Bebek et al., Figure 1B), compared to a 4-fold range in our data (Rn values from 5,000 to 20,000 (Figure 1). This large difference in sample-to-sample variability might explain why the data from some studies (Eid et al., 2005; Lee et al., 2004) and our data showed a significant difference in AQP4 expression whereas Bebek et al., (2013) did not.

Changes in differential AQP4 expression could be implicated in the pathophysiology of sclerosis via water-mediated changes in neuronal activity through AQP4-mediated water efflux at the end-feet (Amiry-Moghaddam & Ottersen, 2003).

Our microarray data have revealed a significant upregulation of AQP1 at the mRNA level. This result is in agreement with the findings reported through the transcriptomics analysis work by Jamali et al., (2006). Normally in the hippocampus, AQP1 is only observed in astrocytes or cerebrovascular endothelial cells under stress conditions. However, in a chronic model of status epilepticus (SE), AQP1 expression was observed in astrocytes
following SE at 4 weeks, and was up-regulated at 6 weeks when a significant up-regulation was reported in epileptic hippocampi compared to control (Kim et al., 2009). In addition, AQP1 expression in cerebrovascular endothelial cells is only highly expressed in conditions where the blood brain barrier (BBB) is disrupted such as in malignant brain tumours (Papadopoulos & Verkman, 2013). There is transient opening of the BBB during SE, which shows epileptogenic effects and also induces inflammation (Kovács et al., 2012; Gorter et al., 2015). Therefore, in drug resistant TLE hippocampi, AQP1 expression could be induced in endothelial cells, which could facilitate water movement across the BBB but this needs to be confirmed and validated using immunohistochemical studies. However, the AQP1 ELISA data reveal that there was no significant difference in AQP1 protein levels between TLE-HS and TLE-NC samples which could be due to various translational regulation mechanisms.

It is well known that regulatory mechanisms underlying AQP gene and protein expression are complex and could be influenced by various physiological, pathological or regulatory stimuli, including hormones (Gu et al., 2003), cytokines (Yang et al., 1995) and/or stress activated signals (Arima et al., 2003). For example, hypertonic stress upregulated AQP1 expression in rodent renal medullary cells by inducing an extracellular signal-regulated kinase including p38 and JNK, which regulate a hypertonicity-responsive element present in the AQP1 promoter (Umenishi & Schrier, 2003). It has been shown that some pathological conditions including multiple sclerosis could result in simultaneous upregulation of AQP1 and AQP4 (Satoh et al., 2007). However this is not always the case, as suggested by the data from our study, which is supported by comparable findings (Mao et al., 2006) showing that inducing severe hydrocephalus stimulated AQP4, but not AQP1, protein expression in perivascular astrocytes.

Restoration of the ion gradients, after seizures, requires energy and an enhanced oxygen and glucose consumption; in fact, it was found that during epileptic activity there is a reduction in levels of glucose and ATP in the tissue as well as an increased level of lactate.
Folbergrová et al., 2000). In order for the neurons to meet this enormous metabolic demand they use glycerol and lactate as a source of energy (Magistretti et al., 1999; Badaut et al., 2014). The aquaglyceroporin, AQP9, is expressed in both astrocytes and neurons in the brain. AQP9 is able to transport both glycerol and lactate, therefore, it may play a vital role in the changes in astrocyte and neuronal energy metabolism that occurs in response to seizure activity (Amiry-Moghaddam & Ottersen, 2003; Badaut & Regli, 2004). In the present study, the total AQP9 transcript level is reduced in TLE-HS compared to TLE-NC indicating that it might be contributing to the pathophysiology of TLE-HS; however, these results need to be confirmed at the protein level in future studies.

DAVID and KEGG analysis of the microarray data identified that 50 of the 3,333 differentially regulated genes were most commonly associated with the MAPK pathway in TLE-HS compared to TLE-NC, as indicated in Figure 4. Interestingly, it has been shown that the p38 MAPK-dependent pathway is possibly the primary mechanism in controlling the altered expression of a number of major AQPs including AQP4 and AQP9 (Fujita et al., 2003) as well as AQPs 3, 5 and 8 (Yang et al., 2013b). This study has identified changes in AQP4 protein expression in human TLE-HS and key regulatory elements of the MAPK pathway by DAVID/KEGG analysis. Our results (Figure 6) reveals that the activity of p38 and JNK MAPKs, but not ERK1/2, contributes to the differential expression of AQP4 protein and hence the astrocytic response to changes in water homeostasis. These results are in agreement with previous reports using cultured astrocytes and in animal models (Rao et al., 2010; 2011). Our results together with evidence of TLE-impaired water homeostasis (Bronen et al., 1991; Lee et al., 2004; Heuser et al., 2010; Dawe et al., 2014) may implicate these elements in the pathophysiology of TLE directly or indirectly through their effect on AQP expression, therefore revealing possible new therapeutic targets. Further studies investigating the effects of p38 and JNK inhibitors on animal models of epilepsy will be needed to validate these findings.
The up-regulation of ERK2 at the genetic and protein levels could be linked to the mechanism underlying the pathophysiology of sclerotic TLE. ERK activation has been reported to induce epilepsy in mouse models by stimulating the N-methyl-D-aspartate (NMDA) receptor. The ERK signalling cascade may contribute to the aetiology underlying some other epileptic types in humans (Nateri et al., 2007). The present study also reveals a significant increase in p53 protein in the sclerotic hippocampi of TLE patients. This is consistent with immunohistochemical data that showed up-regulation of p53 in the hippocampi of TLE patients (Xu et al., 2007) along with a correlation between seizure-activated neuronal death in rat hippocampi and p53 responses (Sakhi et al., 1994; Araki et al., 2004). However, neuropathological studies reveal that seizure-mediated cellular death in TLE happens in a very limited manner (Bernasconi et al., 2002; Mathern et al., 2002; Liu et al., 2003; Engel et al., 2007). This might suggest a pro-apoptotic effect of p53; this study suggested induced activities for several anti-apoptotic and cytoprotective signalling cascades including AKT and RSK. AKT is the key modulator of the AKT-mTOR signalling pathway that is involved in neurogenesis, dendrite development and synapse formation. This result is consistent with the recently published study by Griffin and colleagues (Griffin et al., 2016). RSK modulates mTOR signalling and is involved in cell survival and proliferation in addition to its role in inhibiting the pro-apoptotic function of Death-associated protein kinase 1 (DAPK1) and Bcl-2-associated death promoter (BAD). Furthermore, these cytoprotective and anti-apoptotic properties could also be mediated by inhibition of JNK2 since JNK2 is known to stabilise p53 activity by blocking its ubiquitination (Fuchs et al., 1998; Bode & Dong, 2007); and additionally the inhibition of p38 delta (Zarubin & Jiahuai, 2005).

Differential changes in apoptosis-related signalling cascades are largely found in TLE tissue. In humans, these findings could indicate that seizure-mediated stress could result in alterations in gene expression between adaptive responses that inhibit the neuronal loss and the cell death signalling pathways.
Seizures are usually associated with a high energetic demand due to abnormal simultaneous firing of a large number of neurons. As the seizure progresses, the increased level of lactic acid decreases the tissue pH and causes metabolic-acidosis, which terminates the seizure (Yang et al., 2013a). During seizures, there is a remarkable increase in glycolysis due to inhibited aerobic metabolism; lactic acid is utilised as one of the major sources of energy (Williamson et al., 2005). Previous clinical studies suggest that carbohydrate metabolism and glycolysis could induce susceptibility to epileptic attacks and inhibiting glycolysis could have antiepileptic effects (Huttenlocher, 1976). The results from this study show significant differential expression of a number of essential elements involved in cerebral energy metabolism including the upregulation of the beta isoform of glycogen synthase kinase-3 (GSK3β), but not the alpha isoform. GSK3β is known to act as a negative regulator in the hormonal control of glucose homeostasis and an inactivating agent of glycogen synthase (Cho, 2011). The dual specificity mitogen-activated protein kinase kinase 3 (MKK3) is also upregulated. MKK3 is activated by mitogenic and environmental stress and also by insulin and it is necessary for the expression of the glucose transporter (mainly through its effect on GLUT1 and GLUT4) (Fujishiro et al., 2001; Zarubin & Jiahuai, 2005). Further understanding of the specific role of energy metabolism and homeostasis in TLE could shed light on TLE pathophysiology, and may help in identifying novel therapeutic strategies.

In conclusion, the mechanisms involved in TLE aetiology are complex and currently no single factor that could explain the underlying pathophysiology has been identified. Understanding the signalling networks is essential for discovery and validation of new potential therapeutic targets and we have identified a number in this study using a global transcriptomics approach. Despite the essential role of AQPs in the pathophysiology of many diseases, including cerebral oedema, diabetes insipidus, cancer, TLE and many more, there are still no clinically-available drugs that target AQPs specifically (Verkman, et al., 2014). Therefore, understanding the molecular mechanisms involved in their regulation could provide new insights into the treatment of epilepsy and pharmaco-resistant TLE in particular.
Targeting molecules of the MAPK signalling pathway involved in the regulation of AQP4 expression could be one important area for future studies.

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**Competing Interest**

The authors do not have any competing interest.

**Author Contributions**

MMS, MAS, MTC, and APP designed all experiments. MMS performed and analysed experiments shown in figures 2, 5 and 6. MAS performed the microarray and subsequent analysis shown in figures 1, 3 and 4. MMS performed all the statistics. MMS and MAS equally drafted the manuscript. DB, PK, ACM, RMB, MNW, MTC and APP critically read and revised the manuscript. All authors approved the final version of the manuscript.

**Data Accessibility**

All relevant data are within the paper and its Supporting Information files were made publicly available at: DOI 10.6084/m9.figshare.5100394.

**Abbreviations**
Anti-epileptic drugs (AEDs)

Aquaporins (AQP)

BBB: Blood Brain Barrier

Bcl-2-associated death promoter (BAD)

Central nervous system (CNS)

Cornu Ammonis (CA)

Database for Annotation, Visualization, and Integrated Discovery (DAVID)

Death-associated protein kinase 1 (DAPK1)

Differentially regulated genes (DEGs)

Dentate gyrus (DG)

Electroencephalogram (EEG)

Fold change (FC)

Glial fibrillary acidic protein (GFAP)

Glycogen synthase kinase-3 (GSK3β)

Gene Ontology (GO)

Hippocampal sclerosis (HS)

Kyoto Encyclopedia of Genes and Genomes (KEGG)

Mesial temporal lobe epilepsy (MTLE)

Microarray (MA)

Mitogen-activated Protein Kinase-dependent (MAPK)

Neuronal nuclear antigen (NeuN)
Phosphate Buffered Saline and 0.05% Tween 20 (PBS-T)

RT-PCR (Real-Time Polymerase Chain Reaction)

Sandwich enzyme-linked immunosorbent assay (ELISA)

Sclerotic hippocampi (TLE-HS)

Temporal lobe epilepsy (TLE)

TLE-neocortex (TLE-NC)

References


Tables:

Table 1. Patient clinical data:

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Table 2: Fold change (FC) expression of MAPK target genes and corresponding proteins.

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<th>MAPK Targets</th>
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<th>Phosphorylated MAPK Protein Fold-Change (TLE-HS/TLE-NC)</th>
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<td>AKT Pan</td>
<td>-</td>
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<td>p38 delta</td>
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<tr>
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Table 2: Fold change (FC) was obtained by dividing the mean of the expression level in TLE-HS by the mean of its corresponding TLE-NC expression level. The data represent a comparative analysis for selected MAPK genes (normalized to array signal) and proteins (normalized to array control) in TLE-NC and TLE-HS. The results are presented as mean ± Standard Error of the Mean (S.E.M) for (n=7) for both TLE-NC and TLE-HS in microarray analysis; and (n=3) for both of TLE-NC and TLE-HS in proteome profiler analysis, using patient matched samples. Kruskal-Wallis with Conover-Inman post hoc analysis was used to identify significant differences between samples (*P < 0.05, **P < 0.01, ***P < 0.001 and ****P < 0.0001, ns = not significant). MA: microarray analysis. TLE: Temporal lobe epilepsy. TLE-HS: spiking TLE sclerotic hippocampus. TLE-NC: non-spiking TLE superior temporal gyrus. ns= not significant, - = data not available.
Figure captions:

**Figure 1:** The mRNA expression profile of cerebral AQPs in TLE-HS and TLE-NC specimens investigated using microarray analysis. Histograms represent a comparative analysis for the cerebral AQP mRNA expression profile in TLE-HS and TLE-NC. Kruskall-Wallis with Conover-Inman post hoc analysis tests were used to identify significant differences between samples (*P < 0.05, **P < 0.01 and ***P < 0.001). Data presented as Mean ± S.E.M. Number of samples: TLE-HS (n=7); TLE-NC (n=7). MA: microarray analysis. TLE: Temporal lobe epilepsy. TLE-HS: spiking TLE sclerotic hippocampus. TLE-NC: non-spiking TLE superior temporal gyrus. MA KCNJ10: potassium voltage-gated channel subfamily J member.

**Figure 2:** The translational profile of (A) AQP4 and (B) AQP1 in TLE-NC and TLE-HS specimens investigated by sandwich ELISA. The AQP4 protein level was significantly up-regulated in TLE-HS. The protein level of AQP1 was increased in TLE-HS, though was not statistically significant. Kruskall-Wallis with Conover-Inman post hoc analysis tests were used to identify significant differences between samples (****P < 0.0001). Number of samples: TLE-NC (n = 3); TLE-HS (n = 3) paired samples. All data presented as Mean ± S.E.M. MA: microarray analysis. TLE: Temporal lobe epilepsy. TLE-HS: spiking TLE sclerotic hippocampus. TLE-NC: non-spiking TLE superior temporal gyrus.

**Figure 3:** KEGG Pathway Enrichment Analysis for differentially-expressed genes in TLE-HS vs TLE-NC Data represent KEGG Pathway Enrichment Analysis for differentially-expressed genes (DEGs) in TLE-HS vs TLE-NC. The Enrichment Score value for each KEGG Pathway is reported on the side of each bar and the number of genes identified in each pathway is reported as a number inside each bar.

**Figure 4:** The distribution of the differentially-expressed genes within the detected MAPK signalling pathway in TLE-HS (adapted from KEGG website: www.genome.jp/kegg) The significant up- and down-regulated MAPK related genes, indicated with red and yellow stars respectively, were identified using DAVID/KEGG enrichment analysis of microarray data. The pathway enrichment score was 6.1 and the total number of enriched genes was 50 (27 genes were significantly down-regulated while 23 genes were significantly up-regulated). Number of samples: TLE-HS (n=7); TLE-NC (n=7).

**Figure 5:** MAPK signalling profile in TLE-NC and TLE-HS samples (A) microarray analysis for mRNA expression and B) proteome profiling analysis. Histograms represent a comparative analysis for selected MAPK genes and proteins in TLE-NC and TLE-HS. Kruskal-Wallis with Conover-Inman post hoc analysis tests were used to identify significant differences between samples (*P < 0.05, **P < 0.01, ***P < 0.001 and ****P < 0.0001). Data presented as Mean ± S.E.M. Number of samples: MA (n=7); proteome profiler analysis (n=3). MA: microarray analysis. Rn: Normalized fluorescence intensity. TLE: Temporal lobe epilepsy. TLE-HS: spiking TLE sclerotic hippocampus. TLE-NC: non-spiking TLE superior temporal gyrus.

**Figure 6:** The correlation between p38, ERK1/2 or JNK inhibition and AQP4 protein expression in primary human astrocytes. Data are mean fold-changes in expression (±S.E.M) (n=4). The p38 inhibitor is 10 µM SB203580; the ERK1/2 inhibitor is 10 µM PD98059; and the JNK inhibitor is 40 µM SP600125. FC: fold-change. Each bar represents the normalized mean ± S.E.M for each of the conditions. * represents statistical significance (p < 0.05).
Figures:

Figure 1:
Figure 2:

(A) AQP4

P < 0.0001

****

(B) AQP1

P = 0.0656

Optical Density at 450 nm

TLE-NC  TLE-HS

TLE-NC  TLE-HS
Figure 3: Enrichment analysis for KEGG pathways

- Calcium signalling pathway: 43, score: 8.7
- MAPK signalling pathway: 50, score: 6.1
- Neuroactive ligand-receptor interaction: 48, score: 5.9
- Systemic lupus erythematosus: 21, score: 3.3
- Regulation of actin cytoskeleton: 35, score: 3.1
- Type I diabetes mellitus: 12, score: 3.0
- Graft-versus-host disease: 11, score: 2.7
- Adherens junction: 16, score: 2.5
- Cell adhesion molecules (CAMs): 23, score: 2.5
- Allograft rejection: 10, score: 2.4
- Wnt signalling pathway: 23, score: 1.7
- Long-term potentiation: 13, score: 1.7
- PPAR signalling pathway: 13, score: 1.7
- Axon guidance: 20, score: 1.7
- ErbB signalling pathway: 15, score: 1.6
- Chemokine signalling pathway: 26, score: 1.5
- Hematopoietic cell lineage: 14, score: 1.3
- Apoptosis: 14, score: 1.3
Figure 4:
Figure 5:
(A) Microarray MAPK

(B) Human MAPK Proteome Profiling Array
Figure 6:
Supplementary material:

Supplementary Figure 1:

Supplementary Figure 1: The mRNA expression profile of NeuN and GFAP in TLE-HS and TLE-NC specimens investigated using microarray analysis. Histograms represent a comparative analysis for NeuN and GFAP mRNA expression profile in TLE-HS and TLE-NC. Kruskall-Wallis with Conover-Inman post hoc analysis tests were used to identify significant differences between samples (*P < 0.05, **P < 0.01 and ***P < 0.001). Data presented as Mean ± S.E.M. Number of samples: TLE-HS (n=7); TLE-NC (n=7). MA: microarray analysis. TLE: Temporal lobe epilepsy. TLE-HS: spiking TLE sclerotic hippocampus. TLE-NC: non-spiking TLE superior temporal gyrus.
Supplementary Table 1: Functional annotation clustering of differentially expressed genes (DEGs): Cluster 1-3

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